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CARBON DIOXIDE FIXATION BY GREEN PLANTS

A. A. Benson and M. Calvin

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CARBON DIOXIDE FIXATION BY GREEN PLANTS*

by

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Introduction

Since the end of the war when the long-lived isotope of carbon, C^{14}, became available a new tool has been applied in the study of photosynthesis. Because of the interest evoked by the tracer method, research in all areas of photosynthesis has expanded.

There have been reviews on various aspects of photosynthesis such as the primary photochemical reaction (1), quantum efficiency products, and comparative biochemistry (2), many discussions of which were included in the monograph of The American Society of Plant Physiologists, "Photosynthesis in Plants", (3).

The discovery of the Hill reaction and its elaboration by various workers as well as the work with the tracer carbon has seemed to indicate a definite separation between the carbon assimilation and oxygen evolution aspects of photosynthesis. Since a number of reviews in recent

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years have discussed the oxygen evolution reaction more particularly, we are here going to limit ourselves to the carbon assimilation reactions which are particularly susceptible to study with tracer carbon.

We will, therefore, be concerned with the possibility of answering questions such as: Through what sequence of compounds does carbon pass on its way from carbon dioxide to plant materials? What is the relationship between this process and other anabolic and catabolic processes in the plant?

Before discussing the more recent results of the tracer method it is well to describe some of the work directed toward solution of these problems by the classical methods of gross analysis for a variety of compounds in green plants which have been subjected to various conditions.

Section I

Effect of growth conditions on assimilation.- The effect of growth conditions on the overall composition of cell material has been the subject of much investigation and allows some conclusions to be drawn regarding the pathways of synthesis. Spoehr and Milner (4) have adjusted growth conditions of Chlorella such that the cells contain as much as 85.6 per cent (dry weight) fats. Protein synthesis is favored by high nitrogen nutrition and low light intensity; low nitrogen supply and high light intensity lead to storage of lipids. An empirical method for comparison of degree of reduction (R-value) determined by an elemental analysis was developed by which it is possible to calculate approximate carbohydrate, lipid and protein composition of plant tissue. Burström (5) concluded

previously that nitrate reacts with intermediates of photosynthesis to form amino acids. Myers and co-workers (6,7,8) have studied Chlorella nutrition using the assimilatory quotient (CO_2/O_2) as a tool for interpreting the nature of synthetic processes occuring. Basing his empirical equations for photosynthesis upon the actual elemental composition of cells formed and their nitrogen source, Myers predicted photosynthetic quotients and obtained experimental agreement. In defining the end products of photosynthesis, Myers pointed out that their relationships are determined by the pathways of synthesis and interconversion rather than their state of reduction. Since carbohydrate is the major product of photosynthesis and substrate for respiration in most plants, it has been convenient to consider photosynthesis as a unit process for its production. Myers (6) and others have questioned this viewpoint and feel that the results obtained suggest that photosynthesis merges with plant metabolism prior to appearance of carbohydrates. That these relationships exist is shown in the tracer experiments described later.

Pucher, Leavenworth, Ginter and Vickery (9) have made a careful study on the compounds present in leaves of Brophyllum calycinum as a function of conditions under which the leaves were collected. Increases of starch and hexose content during the day and of total organic acids (largely malic) during the night were observed. Oxidation in the dark via the Krebs tricarboxylic acid cycle seemed apparent to these workers from their results and is comparable to the dark respiration of photosynthetic intermediates discussed below.

The earlier work of Smith (10) had demonstrated that sunflower leaves store sucrose and starch. Brown (11) studied the effect of prolonged photoreduction upon the carbohydrate content of Scenedesmus. He found decreased soluble saccharides and no change in insoluble polysaccharide content after a thirty-hour period of photoreduction. He concluded that the storage products of photoreduction are not carbohydrate, as had been inferred from the assimilation quotient. It appears reasonable that algae should form protein and fats in large amounts for future growth rather than the carbohydrates formed in higher plants as energy sources for translocation to non-photosynthetic tissues. Brown's results might have been more meaningful had he compared algae which had photosynthesized in carbon dioxide with those which had photoreduced carbon dioxide with identical times, light intensities, media, etc.

Absorption of carbon dioxide by isolated chloroplasts.- Reduction of CO_2 has not been observed during oxygen evolution by isolated chloroplasts. Boyle (12) reported that minute amounts of CO_2 are necessary for oxygen evolution but Brown and Franck (13) and Aronoff (unpublished) observed no significant $C^{14}O_2$ fixation under a variety of conditions. Boîchenko (14,15) showed that a hydrogenase system exists in chloroplast preparations obtained from a number of plants, which converts CO_2 in the presence of hydrogen to a substance capable of reducing mercuric ion. The nature of the reducing substance was not determined although its formation paralleled CO_2 uptake. The small extent to which it was formed (10 per cent of the chloroplast weight) leaves some doubt regarding its direct derivation from CO_2.

Metabolism of added intermediates.- Another classical approach in the elucidation of chemical reactions involved in photosynthesis involves feeding suspected metabolic substrates to the intact plant. Experiments such as those of Myers (16) on the oxidative assimilation of acetate and glucose in Chlorella, of Algeus (17) on glycine assimilation in Scenedesmus, and of Kolesnikov (18) on glycolic acid oxidation by barley brei are typical of this approach. Unfortunately cell membranes are not readily permeable to many important intermediates such as phosphate esters and are permeable to carboxylic acids only at low pH. Experiments describing the metabolic fate of isotopically labeled substrates are more conclusive since small amounts of assimilated compounds may be readily studied. Krotkov and Barker (19) studied the assimilation of radioactive acetate and found it taken up into water-soluble substances, prior to respiration as CO_2. Similar dark experiments with tobacco leaves by Tuttle (20) have shown that labeled acetates become incorporated in malic and citric acids in a manner consistent with the existence of a tricarboxylic acid cycle. The carboxyl groups of acetate or glycine were readily respired while the methyl carbon of acetate is respired slowly and that of glycine not at all. From this evidence, acetate was not believed an intermediary per se in normal respiration.

C^{14}-labeled sugars, carboxylic acids and amino acids are increasingly available. A number of such feeding experiments during photosynthesis have been performed in the writers' laboratory and the resulting soluble metabolic intermediates have been separated by paper chromatography. The results obtained provide additional support for the proposed CO_2

assimilation processes. While present methods allow a simple and rapid determination of metabolic products there remains the problem of getting the metabolite into the cell in the biologically active form and proving that it reacts exactly as the natural substrate. Of all substrates, labeled CO_2 is the only one free of such equivocation and its use has produced much of the present knowledge of the path of carbon in photosynthesis.

Intermediates of Carbon Dioxide Reduction

The earliest work with the tracer method was that of Ruben and co-workers which extended over the period of 1938-43. Most of this work has already appeared in several reviews and will be discussed here only insofar as the present results allow its interpretation.

Ruben, Kamen and Hassid (21), examined the products of short photosynthesis (one to five minutes) and found evidence for the absence of many compounds identified in our experiments. Of the compounds Ruben added as carrier, no activity was found in the following: pyruvic, glyceric, succinic, malic, citric, fumaric, aspartic and glutamic acids; alanine, serine and glycine; glucose, fructose and sucrose. When diffusion and sedimentation rates of the radioactive products were measured the molecular weight appeared to be $\sim$ 1000. In the light of present results it is not exactly clear why substances such as malic acid and alanine were not found active. It is possible that adsorption of such compounds on large molecules affected molecular weight determinations. It is now recognized that co-precipitation methods such as those used by Ruben et al. are

unreliable, especially when used to separate more than one substance at a time from the same solution. Unfortunately those workers had not included phosphoglycerate or hexose phosphates as carriers when examining known compounds for radioactivity. Although none of the early intermediates had been identified, Ruben arrived at the conclusion that there was a primary carboxylation reaction to form RCOOH which was followed by a photochemical reduction which regenerated the carbon dioxide acceptor RH and thus resulted in carbon dioxide assimilation. He assumed that carbon dioxide was fixed anaerobically in the dark by Chlorella in a compound, RCOOH, which was subsequently reduced during photosynthesis. The first available C^{14} was used in an effort by Ruben and one of us to isolate this compound. This work was continued at Berkeley after the war with the ultimate isolation of succinic acid (22) as the major product. Succinate had been identified as a $C^{11}O_2$ fixation product by the protozoan, Tetrahymena geleii by van Niel et al. (23) and in the fermentation of glycerol in $C^{11}O_2$ by Propionibacterium pentosaceum by Carson and Ruben (24).

Determination of the path of carbon in photosynthesis.- In view of the fact that both Ruben and others had demonstrated the fixation of CO_2 in the dark by a variety of organisms it appeared desirable to perform experiments in such a way that the method of carbon dioxide fixation was unequivocally that of photosynthesis. This quite clearly entailed the feeding of radioactive carbon dioxide to the organisms in an active state of photosynthesis in the light and studying the sequence of intermediates through which the carbon passes. The results of such experiments have so

far been published by four groups of workers and will be discussed from the point of view engendered by our own work.

The algae** or leaves are allowed to photosynthesize in normal CO_2. Radioactive sodium bicarbonate or $C^{14}O_2$ is rapidly added in an amount small enough not to disturb steady state concentrations of metabolites. The partial pressure of CO_2 should change little during the experiment. After the chosen period the plants are killed as rapidly as possible, usually by boiling ethanol.

In short photosynthesis experiments (less than one minute) almost all of the radioactive products are soluble in 80 per cent ethanol (25). Brown, Fager and Gaffron (26) have reported complete curves for the relative amounts of $C^{14}O_2$ converted to fats and benzene-soluble pigments (fraction A), alcohol soluble compounds (fraction B) and insoluble material such as denatured proteins, cellulose and starches (fraction C). These workers did not state the probable components of their extracts and residues. In their shortest exposures to $C^{14}O_2$, all of the fixed radioactivity was found in fraction B.

Separation of intermediates.- Ion exchange resin separations (22,25,27,28) allowed the separation and identification of alanine, phosphoglyceric acid, and equal amounts of glucose and fructose as some of the products of

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** We are indebted to Professor Gaffron for the strain of Scenedesmus (D_3) used in this work.

short photosynthesis experiments. Phosphoglycerate is unique among the major products of short photosynthesis in that its two acidic groups increase its affinity for the anion exchange resins. It is less readily eluted than any other compound stable toward mild acid hydrolysis. This property was used to obtain almost pure samples for chemical comparison with authentic phosphoglycerate.

Silica gel partition chromatography has been applied by Bassham (25) and by Stutz (29) for the separation of carboxylic acids formed by plants during photosynthesis in $C^{14}O_2$. Both reported major fractions of activity incorporated in malic acid. The results reported by Fager (30) indicate that malic acid was present but was not identified.

Burris, Wilson and Stutz (29) reported specific and total activities in carboxylic acids formed from $C^{14}O_2$ during both light and dark assimilations by Bryophyllum, tobacco, tomato and barley leaves. The interconversion of these products in a subsequent dark period was reported. Malic acid was not observed to decrease while citric and isocitric acids increased markedly in the dark. There appeared to be no conversion of malic acid to citric acid. This would seem to indicate that activity in these respiration intermediates is derived from sources other than the carboxylic acids formed in the light. In 30 minutes photosynthesis by barley, 84 per cent of the carboxylic acid activity appeared in malic acid with less than 10 per cent in any other carboxylic acid. In a fifteen minute dark fixation the products included 43 per cent succinic acid and 47 per cent malic acid as well as over twice as much

citric acid and isocitric acids as in the light experiment. These results seem to be added evidence that newly reduced carbon does not rapidly accumulate in respiration intermediates in the light and that malic acid is probably closely related to the intermediates of photosynthesis as well as being involved in respiratory reactions.

Paper partition chromatography (31) of plant extracts for determination of amino acid constituents (32), sugars (33) and protein hydrolysates have been reported. Fink and Fink (34) applied autoradiography in detecting radioactive products on paper chromatograms of compounds synthesized by Chlorella. Since their algae photosynthesized for four hours in $C^{14}O_2$ the products are not directly pertinent to a discussion of the mechanism of CO_2 reduction.

Stepka, Benson and Calvin (35) reported the results of similar techniques on extracts which had photosynthesized for 30 seconds or had fixed $C^{14}O_2$ in the dark immediately after preillumination. In both cases alanine and aspartic acid were found to be the major radioactive amino acids. No labeled glutamic acid was found.

Paper chromatography allows rapid and complete separation of many other compounds involved in photosynthesis (35,36,37). The activity in each substance may be determined by counting the area defined by the radiogram (radioautograph of paper chromatogram) with a large window Geiger-Müller tube. Data so obtained were tabulated by the writers (38).

In determining the sequence of appearance of intermediates it will be helpful to plot activity in each intermediate as a function of

time. As each reservoir becomes saturated with radioactive carbon the slope of the plotted curve will approach zero. Comparison of a set of such curves should be evidence for the sequence of synthesis. In order to simplify the study of synthetic sequence low temperature and low light intensity should slow down the processes involved and simplify the experimental problem.

Phosphoglyceric acid is the major product in experiments of shortest duration (five seconds). A small amount (25 per cent) of phosphopyruvate appears in all cases. Which of these compounds results from addition of $C^{14}O_2$ to a two-carbon acceptor is a subject for investigation of the type discussed in the preceeding paragraph. Triose phosphate has been observed in 15 second experiments while in longer exposures its percentage of total activity becomes small. The total amount of hexose diphosphate observed is also considerably smaller than that of phosphoglycerate even after several minutes. Fructose- and glucose-6- phosphates have not yet been separated satisfactorily. It is known that these compounds lie in an area on the chromatogram containing hexose monophosphates.

The sucrose synthesized in 30 seconds by Chlorella was hydrolyzed and the specific activity of fructose was found twice as high as that of glucose (36). This suggests that synthesis of fructose structures precedes synthesis of glucose phosphates. In Chlorella after 90 seconds the two hexoses in sucrose have nearly equal activity.

The nature of the reactions immediately prior to liberation of free sucrose is not yet known. No free hexoses appear in five minute experiments with algae, sugar beet, barley, or geranium. It is required

then, that sucrose is formed either by simultaneous condensation and dephosphorylation of two hexose phosphate molecules or by dephosphorylation of a sucrose phosphate. No sucrose phosphate has yet been detected.

High molecular weight products.- A number of dextrins have been observed, particularly in experiments with Bryophyllum leaves and algae. Algae which have photosynthesized for two minutes and then are starved anaerobically for CO_2 in the light for three minutes before killing have been observed to form a considerable quantity of such dextrins. While their molecular weight is not yet investigated there is evidence that they are simple two, three, four and five glucose structures.

The major portion of insoluble products formed in the first few minutes by algae is protein. Acid hydrolysis produced radioactive amino acids in approximately the same relative amounts as exist in the cell extract. Protein obtained from longer experiments (five to ten minutes) contains larger amounts of amino acids not present in appreciable amounts in the cell extracts. Glutamic acid, by far the largest free amino acid reservoir, is not converted into protein in amounts commensurate with its concentration. The early insoluble products formed by barley are largely carbohydrates as shown by their hydrolysis to glucose and levulinic acid.

Chemical identification.- The methods of chemical identification of these intermediates have been reported in a series of papers from this laboratory (25,28,37). The radioactive products were separated at first by virtue of their absorption properties on exchange resins and identified by chemical properties, distribution coefficients and co-crystallization.

Carboxylic acids such as malic, succinic, fumaric, glyceric and glycolic acids were identified by co-chromatography with synthetic radioactive acids (37). The major compounds involved in sucrose synthesis were shown to contain phosphorus by comparison with the compounds photosynthesized in radiophosphate. They included some of the known glycolysis intermediates which were prepared with radiophosphate. Paper chromatographic separation of phosphate esters has been described by Benson et al. (37), Cohen (39) and by Hanes and Isherwood (40). In addition to co-chromatography of 3-phosphoglycerate with that formed by plants the direct isolation from extracts of Scenedesmus has been described (37). Over 65% of the radioactivity fixed by Scenedesmus in five seconds was isolated in the barium salt of phosphoglyceric acid. Periodate oxidation of glyceric acid formed by enzymatic or acid hydrolysis gives the required amount of activity in formaldehyde, formic acid and CO_2 or in glyoxylic acid and formaldehyde.

Fager (30) has reported results of tests on the radioactive components of fraction B as defined by Brown, Fager and Gaffron (26). It is seen that this water-soluble fraction contains a multitude of compounds involved in photosynthesis and that very few occur to the extent of more than ten per cent in the mixture. Fager proceeded to concentrate the major photosynthetic intermediate with several assumptions in mind. The first was the assumption that the major portion of tracer fixed was in only one or two compounds which are not related to normal respiration intermediates. The second was that the compound is resistant to movement into the rest of the cell by respiration or other metabolic reactions.

The argument for simple compostion of fraction B (26) (40 second photosynthesis) rests in the apparent non-sigmoid character of the C^{14}-in-fraction B assimilation curve. They observed no appreciable lag in formation of fats and insoluble material. The adsorption of low-molecular intermediates on proteins may have caused the appearance of activity in the insoluble material in the shortest experiments. Our experience is that phosphoglycerate is such a strongly adsorbed compound. These workers did not expect that even in experiments as short as 40 seconds one might obtain a large number of radioactive intermediates. The sigmoid character of the curve for fraction B curve might appear only when investigated at shorter times and when the actual nature of the insoluble activity in fraction C is known.

The uniqueness of "B" in metabolic reactions was assumed from the fact that activity in fraction B is not converted to fats or insoluble material in the dark. These conversions are not the only possible results of changes in radioactivity of fraction B. The phosphorylated intermediates of hexose synthesis are rapidly converted to sucrose and to the Krebs tricarboxylic acid cycle intermediates of respiration. With algae, sucrose is respired while in barley leaves the degradation of sucrose is very slow compared to that of the intermediates of its synthesis. It is thus apparent that the simple nature (i.e. one or two compounds) of the radioactivity in fraction B and the resistance to transformation (stability) of this "compound B" save under the influence of light, need not be inferred from its non-conversion to insoluble material in the dark.

In the course of determining the chemical properties of "B",

Fager carefully examined all the reasonably expected metabolic intermediates for radioactivity. Of these none was found radioactive. The major difficulty in the isolation work reported seems to us to have been the dilution of a small amount of radioactivity with tremendous amounts of plant material. Experiments such as the benzoylation of amino acids are typical. An active extract containing 1960 cpm. of C^{14} was benzoylated and 663 mg. of products was examined for radioactivity. The average specific activity, 3 cpm. per mg., is very low compared to the maximum obtainable, 10^6 to 10^7 cpm/mg., and impedes identification procedures. The amino acids present would have given such small amounts of benzoyl derivatives that they would have been water-soluble and would not have appeared in the inactive precipitate reported by Fager. Only solvent extraction of benzoylamino acids which yielded an active extract seems of significance and indicates 15% of amino acids which corresponds to our experiments. Silica gel partition chromatography was used by Fager and indicates possible 23 per cent of dicarboxylic acids such as malic. The evidence presented for the absence of known metabolic intermediates cannot be accepted as precluding the existence of moderate amounts of many of them.

Degradation of intermediates and products.- The distribution of labeled carbon within the molecule is valuable evidence for defining the path by which the compound was formed. Such evidence has been applied in the study of a great number of biochemical processes. Any proposed reaction mechanism must be verified both by the relative sequence of appearance of the intermediates and by the distribution of newly assimilated carbon within the

intermediate. The degradation data which have accumulated in the past few years are the basis of the proposed reaction sequence for photosynthesis given below.

The hexose degradation method developed by Wood, Lifson and Lorber (41) has been widely applied (42,43,44). Lactic acid fermentation followed by chemical degradation gives the relative isotope concentrations in the 3-4, 2-5 and 1-6 pairs of carbons. No evidence has been reported for asymmetric distribution of newly incorporated carbon. Wood and Burr (45) applied this degradation procedure to hexose synthesis by bean leaves from $C^{13}O_2$ and found some variations in C^{13} content. Aronoff, Barker and Calvin (42) reported unequal distribution of C^{14} (61%, 24%, 15% C_3-4, 2-5, 1-6 respectively) in barley hexoses formed during 40 minutes photosynthesis. No satisfactory explanation for unequal C^{14} distribution in an experiment as long as 40 minutes has yet been advanced. Similar results were obtained for hexoses synthesized in 30 seconds (25).

Gibbs (43) reported results of degradation of sucrose isolated after long photosyntheses by canna and barley in which the hexoses were uniformly labeled. However, with monosaccharides (sucrose was not subject to the fermentation) from one hour photosynthesis by barley in $C^{14}O_2$ Gibbs found a distribution of 15%, 28%, 56% in the C 3-4, 2-5, 1-6 positions of the hexose respectively. Such a distribution is most probably caused by a subsequent short period of photosynthesis in $C^{12}O_2$ during the time of opening the photosynthesis chamber and killing the plants. Such results have been obtained previously (25) as a result of photosynthesis of low

specific activity CO_2 at the end of a long experiment. Gibbs (46) found a distribution of 51%, 30%, 19%, in C 3-4, 2-5, 1-6 in hexoses of sunflower leaves which is in agreement with that of Aronoff for 90 second photosynthesis by soy bean leaf (49%, 23%, 19% in C 3-4, 2-5, 1-6) and with the results obtained in the writers' laboratory.

It has become apparent that algae and the higher plants differ considerably in the rate at which hexoses become uniformly labeled. The sequence of labeling, however, is the same in all plants investigated. The hexoses and phosphoglycerate synthesized by barley leaves in periods longer than 60 seconds are uniformly labeled. For Scenedesmus uniform labeling requires three to five minutes.

In five second photosynthesis by Chlorella or Scenedesmus five per cent of the C^{14} in phosphoglycerate is found in the α and β carbon atoms. Barley has 15 per cent in the α and β carbons of phosphoglycerate after only two seconds of photosynthesis. The distribution of C^{14} in alanine corresponds to that in phosphoglycerate and in the hexoses. When malic, succinic or aspartic acids were degraded the distribution corresponded to that derived by addition of $C^{14}O_2$ to a three-carbon compound, as in the Wood-Werkman reaction. Both carboxyl groups are labeled with high specific activities and the central carbon atoms are labeled to the extent of that found in the α and β carbons of three-carbon compounds or in the 2-5 and 1-6 positions of the hexose.

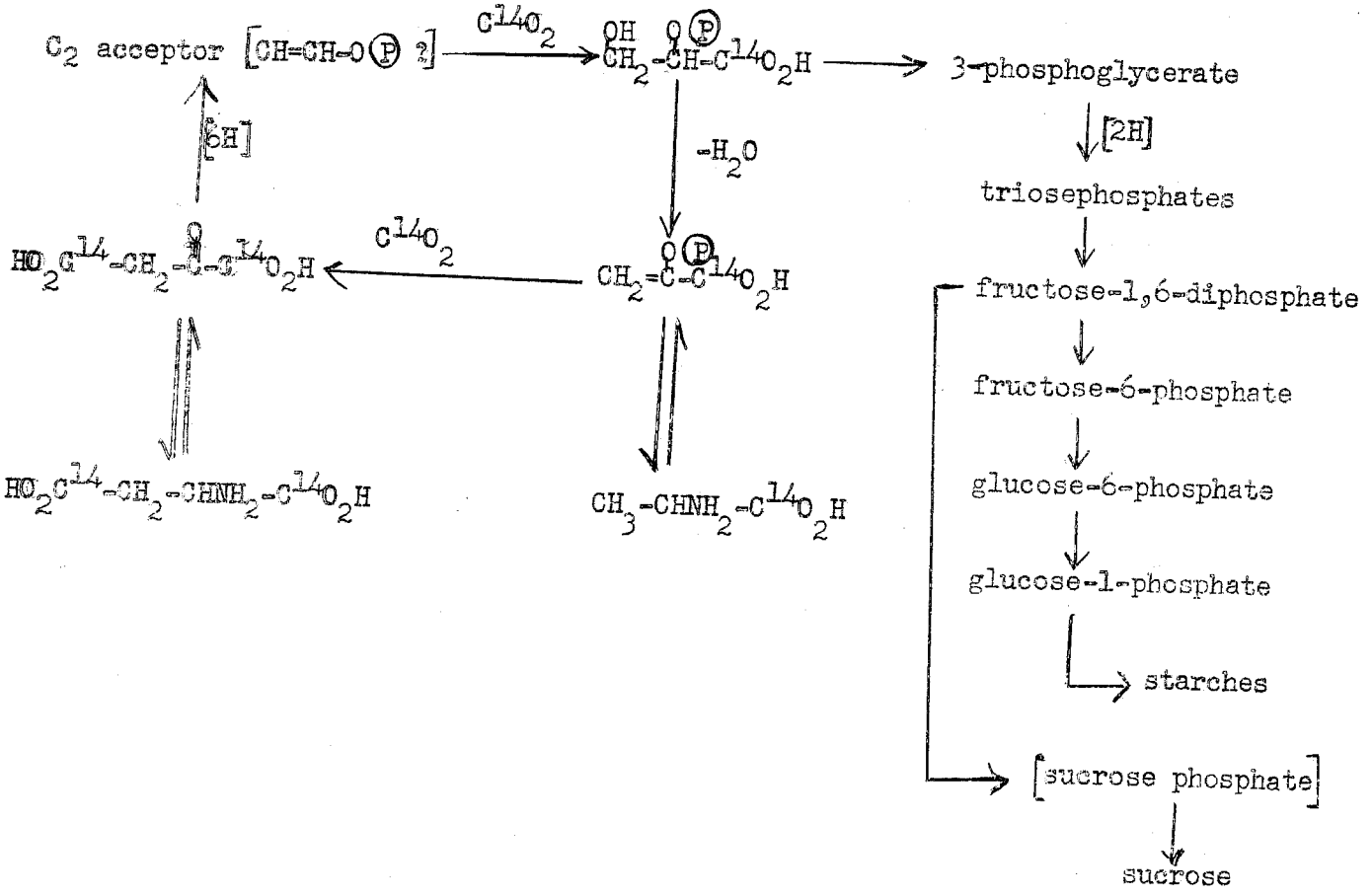
A mechanism has been proposed to account for the observed compounds and their isotopic distribution. It is essentially a dicarboxylic

acid cycle in which a two-carbon acceptor molecule is converted to oxalacetate by two successive carboxylations. Upon splitting the four-carbon acid, two new acceptor molecules are formed. The intermediates of photosynthesis are diverted from this cycle for synthesis of fat, amino acids and carbohydrate.

Cycle Scheme

The phosphoglycerate formed initially is carboxyl-labeled. When a carboxyl-labeled structure has passed through the cycle once, the newly-formed acceptor is labeled in the reactive end. Subsequent α-carboxylation gives phosphoglycerate labeled largely in the carboxyl but also in the α position to an extent determined by the size of the reservoirs of intermediates in the cycle and by the duration of the synthesis. A second passage around the cycle, followed by α-carboxylation yields phosphoglycerate labeled with increased amounts of isotope in the β, α and carboxyl carbons. When such a compound is converted to sucrose similar isotope distribution occurs in the 1-6, 2-5 and 3-4 carbon atoms, respectively, of the hexoses. The sizes of the reservoirs of intermediates in the cycle, as well as those in rapid equilibrium with intermediates greatly affect the rate of attainment of uniform isotope distribution.

The proposed cycle is written without specifying the exact nature of the conversion of oxalacetate to two-carbon acceptor. Originally (22) it was assumed that oxalacetate might be converted by well-known reactions into acetate through malate, fumarate and succinate and a reductive splitting of succinate. The apparent absence of succinate and fumarate in the synthetic paths used by higher plants led to serious doubts regarding their participation.


C2 acceptor [CH=CH-O(P) ?]
C14O2
CH2-CH-C14O2H
3-phosphoglycerate
[2H]
triosephosphates
fructose-1,6-diphosphate
fructose-6-phosphate
glucose-6-phosphate
glucose-1-phosphate
starches
[sucrose phosphate]
sucrose
-H2O
[2H]
HO2C14-CH2-C-C14O2H
C14O2
CH2=C-C14O2H
HO2C14-CH2-CHNH2-C14O2H
CH3-CHNH2-C14O2H

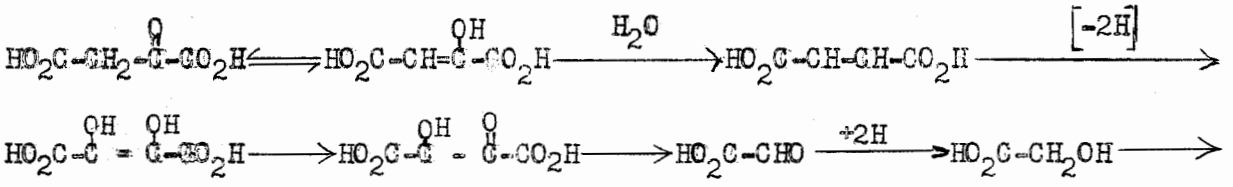
The role of glycolic acid in plant metabolism.- Kolesnikov (47,48) has shown that glycolic acid may act as a substrate for oxidation by suspensions prepared from barley leaves. He also observed a catalytic oxidative effect of glycolic acid similar to that reported earlier by Anderson (49) for the colorless alga Prototheca Zopfli. Kolesnikov (18,48) presented evidence that organic peroxides responsible for such oxidation were formed from glycolic and glyoxylic acids and that both acids acted catalytically in the oxidation of chlorophyll. The observation that this oxidative system was obtainable only from chlorophyll-bearing tissues led him to suggest that it is an element of the photosynthetic system.

Tolbert, Clagett and Burris (50,51) have purified a widely distributed enzyme obtained only from chlorophyll-containing tissues which catalyzes oxidation of l-α-hydroxy acids. Glycolic acid is oxidized via glyoxylic acid to formate and CO_2. Lactic acid is oxidized to pyruvic acid. With crude enzyme preparations or tobacco leaf sap the oxidation proceeds through glyoxylic acid, but to products other than formate and CO_2.

Glycolic acid has been observed as a product of short photosynthesis by all plants studied in the writers' laboratory (38) and by those studied by Burris, Wilson and Stutz (28) excepting Bryophyllum. It appears as a major product ($\sim$ 25 per cent of total radioactivity) during strong illumination, low carbon dioxide pressure and aerobic conditions. Conditions such as these for optimal glycolic synthesis correspond to those for photooxidation described by Franck and French (52) who suggested formation of a carboxylic acid during the process. Glycolic acid is also observed in barley extracts

after photosynthesis in $C^{14}O_2$ followed by a period of illumination with nitrogen flushing. Since glycolic acid is found at low oxygen pressures, even though it be in smaller amounts, one is led to suspect its participation in the synthesis of the two-carbon acceptor of photosynthesis. The appearance of glycine simultaneously with that of glycolic acid, lends support to the participation of glyoxylic acid as well. It remains to be demonstrated conclusively whether glycolic acid arises as a reservoir for two-carbon acceptor molecules or whether its presence is wholly due to photooxidation of such acceptors.

The appreciable amounts of glycine and glycolic acid formed even in short experiments suggested the possibility that the splitting occurred in a four-carbon acid more oxidized than succinic acid. Dihydroxymaleic acid, the oxidation of which was investigated by Kuzin and Doman (53) may play a role in the process but neither this acid nor its precursor, tartaric acid, has been isolated or identified. This possible mechanism (54) is given below.



A path such as this seems reasonable on the grounds that it involves only simple reversible hydrations, hydrogenations and formation of carbon-carbon bonds by benzoin-type condensations, all of which are well-known

in biosynthesis.

The works of Anderson, Kolesnikov, and Clagett, Tolbert and Burris are in agreement with the writers' results and those of Burris, Wilson and Stutz showing that radioactive as well as inactive glycolic acid disappears rapidly in the dark. This rapid disappearance before the plant is killed accounts for the previous absence of conclusive identification of glycolic acid in plants.

Dark Fixation of Carbon Dioxide

The dark fixation of $C^{11}O_2$ in Chlorella and barley was observed by Ruben, Hassid and Kamen. These experiments have been repeated by Brown, Fager, and Gaffron (26) and by Benson et al. (37) with Scenedesmus. The results of the three groups are nearly identical except that Ruben's curve (21) showed that dark fixation reaches a saturation point. Actually a slow dark uptake proceeds for many hours. The Chicago group confirmed the earlier finding that such dark fixation is a reversible process but discounted Ruben's supposition and that of Allen, Gest and Kamen (55) that such dark fixation is related to photosynthetic fixation except for some possible very unstable and rapidly dissociable product.

These experiments are readily interpreted in view of the nature of products formed (38). Chlorella, Scenedesmus and barley incorporate $C^{14}O_2$ in the dark into carboxyl groups of glutamic, citric (iso), succinic, fumaric, aspartic and malic acids as well as in alanine. These compounds, related to or members of the Krebs tricarboxylic acid cycle, are apparently

universally involved in plant as well as in animal respiration as is shown by the works of Bonner, Vennesland, Weinhouse and others. The rate of dark fixation is constant except for an initially rapid uptake, presumably caused by previous depletion by pumping or by flushing with a CO_2-free gas.

Carbon dioxide fixation by succulents. - Bonner and Bonner (56) determined the rate of acid accumulation in leaves of several succulents as a function of temperature and carbon dioxide-pressure. They observed a rapid linear rise in acid accumulation to 0.1 per cent carbon dioxide followed by a much slower linear rise up to concentrations of 10 per cent carbon dioxide, not unlike the effect on photosynthetic rate of other plants or the effect on dark fixation rate of preilluminated Scenedesmus (57). The nature of the acids formed during the relatively large scale dark fixation of $C^{14}O_2$ by Bryophyllum crenatum was determined by Bonner and Thurlow (58). The amount of radio-carbon fixed in the leaves was nearly twice that simultaneously respired and twice the net fixation. The fraction including isocitric, citric and malic acids contained half the fixed activity and are the same compounds as those observed in dark fixation by algae and barley (38).

Enhancement of dark fixation by preillumination. Seeking enhanced dark fixation for isolations of the compounds formed, the writers (28,37) illuminated plants anaerobically in the absence of carbon dioxide. The result was a ten- to one hundred-fold increase in initial dark fixation rate followed by a slow normal fixation rate. The dark fixation ability increased to a maximum with preillumination. Scenedesmus required one or two minutes

of preillumination to reach maximum dark reduction ability; Chlorella was several times slower. This phenomenon was interpreted (28) as a formation of "reducing power", such as a reduced coenzyme, or any other reduced compounds by preillumination which could later reduce a limited amount of CO_2 in the dark. This reducing power was observed to decay in the dark ($t_{1/2}$ = ~2 minutes) due presumably to reduction of respiratory or fermentation CO_2 or other reducible substrates, and could be repeatedly restored by illumination. It should be pointed out that this phenomenon is diminished at very high light intensities.

This interpretation has been criticized by Brown, Fager and Gaffron (26) and by Franck who interpret the result as a restoration of equilibrium shifted by photosynthetic depletion of carbon dioxide during preillumination. The analyses of Calvin and Benson (28) show considerable increase of activity in alanine after dark fixation, as a function of preillumination. However, radioactive glutamic and citric acids are not formed under these conditions. Thus the depletion and restoration of the alanine reservoir by preillumination and dark fixation does not indicate shifts in respiratory intermediates, but rather in photosynthetic ones. Most convincing evidence for the direct relationship between preilluminated dark fixation and photosynthetic fixation lies in the identity of compounds formed in the two cases (38). Considerable quantities of sucrose and intermediates in its synthesis have been formed in the dark by preilluminated Chlorella and barley leaves. Most of the CO_2 absorption occurs in the first half minute of such fixation. More time, from one to two minutes,

is required for sucrose synthesis from its phosphorylated percursors.

The possibility that enhanced dark fixation could have been due to a mass action reversal of respiratory or fermentative decarboxylations was considered by the writers (57). The dependence of dark fixation on carbon dioxide pressure was determined for normal and preilluminated Scenedesmus. The curve for preilluminated cells, which resembled that for the dependence of photosynthetic rate on carbon dioxide pressure, had an initial slope one hundred times greater than that for dark fixation by normal cells. Because of compensation by fermentation or respiration the carbon dioxide partial pressure within the cell cannot reasonably be expected to decrease one hundred-fold (i.e. to 0.001 mm) during preillumination it was concluded that the major action of the light was to produce reducing agent(s) and carbon dioxide acceptor(s).

The distribution of C^{14} within the intermediate molecules also shows the nature of this dark fixation. Only carboxyl-labeled compounds would be expected from reversal of known fermenation or respiration reactions. Degradation data accumulated in this laboratory (25) demonstrate the presence of four to ten per cent of C^{14} in carbons other than carboxyl groups in alanine, phosphoglycerate and succinate, as well as in the 2,5 and 1,6 carbons of the hexoses. While it is possible that such a result is due to some sequence of side reactions as yet unknown, the writers feel that such labeling may best be explained as an actual operation of the CO_2-acceptor regenerating cycle described earlier.

Differential inhibition of respiration and dark carbon dioxide-fixation.-

The earlier cyanide inhibition experiments of Ruben, Kamen and Hassid (21) were extended by Allen, Gest and Kamen (55) who used both Chlorella and Scenedesmus. This work has been criticized extensively by van Niel (3) who concluded that the evidence presented does not preclude photosynthetic CO_2 fixation via some reversible step in the Krebs cycle. The magnitude of the dark $C^{14}O_2$ fixation in the presence of cyanide was shown to parallel photosynthetic activity and to be independent of endogenous respiration in both algae. Previous starvation reduced dark fixation by Scenedesmus but not by Chlorella. These results did not convince Brown, Fager and Gaffron (26) that the dark fixations observed were in any way related to the dark pick-up which must precede photosynthesis. They suggested that dark fixation by cyanide-inhibited cells is comparable to anaerobic dark fixation and to at least the first stages of aerobic dark fixation.

While no conclusions may definitely be drawn regarding the influence of cyanide on the processes of dark fixation, identification of the various products of aerobic and anaerobic dark fixation shows that they are largely intermediates of the tricarboxylic acid cycle. Experiments performed in the writers' laboratory show that Chlorella and Scenedesmus differ in that Chlorella forms no appreciable amount ($<$ two per cent) of phosphorylated glycolysis intermediates while Scenedesmus forms up to 24 per cent of radioactive phosphate esters during a 40 minute dark fixation. Under aerobic conditions the amount of phosphates and succinic acid is small while the fraction of glutamic acid is large (20 per cent). Succinic acid is the major anaerobic dark fixation product while under aerobic conditions in the

dark succinic, malic and glutamic acids are major products. The major products common to both dark and photosynthetic fixations are alanine, aspartic acid, and malic acid. It should now be possible to compare experimentally the products of dark fixation by normal and cyanide-inhibited Scenedesmus. It may well be that uptake of C^{14} into tricarboxylic acid cycle intermediates may continue while all incorporation into phosphates involved in carbohydrate synthesis is inhibited. Such a result would be in fair agreement with the results of Allen, Gest and Kamen and would be added evidence that all reactions from carbon dioxide to carbohydrate are reversible and require no simultaneous photochemical step.

Relation of respiration to photosynthesis.- All experiments designed to measure the rate of light respiration have involved various assumptions. The selective poisoning experiments of Gaffron (59) with Scenedesmus have their counterpart in those of Warburg (60) with Chlorella. It seems unlikely that true selective poisoning of either photosynthesis or respiration can be obtained since the intermediates and types of reactions are so closely related.

Experiments at low CO_2 pressures where gas exchange at constant light intensity may be extrapolated to zero CO_2 pressure to determine the light respiratory rate have been performed by Hoover, Johnston, Brackett (61), Gabrielsen (62) and Warburg et al. (63). Such experiments have not yet yielded reliable data for extrapolation. Gabrielsen (64) measured the CO_2 content of gas which rapidly flowed past illuminated sun leaves (thick) and shade leaves (thin) of elderberry. Although the shade leaves were found to evolve about as much CO_2 in the light as in the dark the respiration of sun leaves was

reduced about one-half in the light. Gabrielsen interpreted the decrease as due to reassimilation of respired CO_2 since the amount re-photosynthesized diminished with increasing gas velocity and decreasing respiratory rate. If this were true it would mean that respiratory intermediates must be converted to CO_2 before reassimilation by photosynthesis. For similar reasons, Warburg et al. (65) found it necessary to agitate their algae violently in order to prevent reassimilation of respiratory CO_2. Kok (66) measured oxygen exchange near the compensation point as a function of light intensity and extrapolated the light dependence of photosynthesis to zero intensity. As Weigl (67) has pointed out, Kok's data from which he concluded that light respiration was one-half that in the dark could be interpreted to show an increase of respiration at high light intensities. It is difficult to extrapolate from measurements at extremely low CO_2 pressures or light intensities to the relationships prevailing under more normal conditions.

Weigl (67) fed isotopic CO_2 to barley leaves and followed the dilution of the gas phase radioactivity by inactive CO_2 from respiration. He found that light decreased the respiratory evolution of CO_2 but was unable to show whether this was due to a real decrease in the ratio of respiration or merely another case of re-assimilation of the carbon dioxide before it reached the gas phase. It would seem that gas phase measurements alone cannot give a true value for either CO_2 assimilation or respiration in the light.

The question of the availability of photosynthetic intermediates for respiration has also been partially answered by his experiments (67). Intermediates formed from $C^{14}O_2$ were not respired while the light was on.

As soon as the light was turned off the specific activity of the gas rose rapidly, indicating that the newly formed intermediates were being respired. Re-illumination reduced the specific activity as well as the total CO_2 to a low value. The rise in specific activity of the gas phase could again be observed when illumination ceased. These results are in accord with results observed on radiograms of plant extracts (38). When the light is on, whether CO_2 is present for photosynthesis or not, the intermediates of previous photosynthesis with $C^{14}O_2$ are only slowly respired through tricarboxylic acid cycle intermediates. However, when illumination is decreased or ceases, the radioactive intermediates of sucrose synthesis are rapidly respired through the tricarboxylic acid cycle.

In his interpretation of the contradictory results in quantum yield measurements Franck (68) proposed that two entirely different photosynthetic processes may occur. He concluded that chloroplast membranes may be permeable to respiratory intermediates at low pH and impermeable at high pH. The experiments performed in the writers' laboratory with a variety of leaves and algae at pH 4 and with algae up to pH 8.7 have shown no detectable changes in the nature of the intermediates. It is unlikely that such changes in media affect the pH near the chloroplasts as much as do changes in light intensity.

Concluding Remarks

It is clear that with the advent and development of the tracer method for following carbon in plant metabolism the means for determining the detailed and manifold reactions through which carbon passes into the structure of the plants is at hand. Although some progress in this direction has been

made, considerably more time and the efforts of many more laboratories will be required before a clear picture will be obtained of the chemical reactions and interrelations taking place in plants. However, it is not to be expected that this type of work will lead directly to a solution of the unique problems of photosynthesis, namely, the knowledge of the act or acts by which electromagnetic energy is transformed into chemical energy. As a result of these studies with tracer carbon we now believe that the solution of this problem is more likely to be found in investigations of the photochemical production of oxygen by isolated chloroplasts (grana) in the presence of suitable oxidizing agents.

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